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In Vivo Suppression of Osteoclastic and Osteoblastic Activities of Goldfish Scales in Water Containing Cadmium

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The fish scales are well known as a major source of internal calcium storage. Therefore, we developed an original assay system using goldfish scales in which both osteoclasts (bone resorption cells) and osteoblasts (bone formation cells) coexist. In our bioassay system, we utilized the activities of tartrate–resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) as respective indicators of each activity in osteoclasts and osteoblasts. Using this bioassay system, the influence of cadmium chloride ($CdCl_2$) on osteoclastic and osteoblastic activities in the scales of goldfish was examined in an *in vivo* experiment. Goldfish were kept in tap water containing $CdCl_2$ (10^{-7} M) for 2 days. TRAP activity in the scales of goldfish decreased with 2 days of exposure to $CdCl_2$. This inhibitory effect for osteoclasts continued even at 4 days of exposure to $CdCl_2$. In the case of osteoblasts, $CdCl_2$ inhibited ALP activity in the goldfish scales at 4 days after exposure, although ALP activity in the goldfish scales had not changed after 2 days of exposure to $CdCl_2$. In *in vitro* cultured goldfish scales, we previously reported that ALP activity decreased after exposures of 64 and 96 hrs, although their activities did not change after 6, 18, and 36 hrs. These results were supported by our *in vivo* experiment. This is the first report to indicate that both osteoclasts and osteoblasts in fish were suppressed by cadmium (Cd) treatments *in vivo*. Considering both *in vivo* and *in vitro* experiments, we concluded that Cd inhibited both osteoclastic and osteoblastic activities in goldfish. This suggests that Cd leads to a disturbed calcium metabolism and then induces bone anomalies.

Key words: cadmium, *in vivo*, osteoclasts, osteoblasts, goldfish scales

INTRODUCTION

Heavy metals such as cadmium (Cd), mercury, and copper are known to be toxic to organisms. Cd has been identified as a causative substance for ITAI–ITAI disease in Toyama Prefecture, Japan (Tsuchiya, 1969). This disease, caused by Cd, induces nephrotoxicity and osteomalacia and disrupts human bone metabolism (Tsuchiya, 1969). In fish, as well as in humans, the toxicity of Cd is also well known (Brown *et al*., 1994; Hiraoka and Okuda, 1984; Pragatheeswaran *et al*., 1987; Rai *et al*., 2009; Verbost *et al*., 1987). The exposure of fish to Cd disturbs their calcium metabolism, resulting in hypocalcemia (Giles, 1984; Rai *et al*., 2009), followed by bone anomalies (Hiraoka and Okuda, 1984; Muramoto, 1981; Pragatheeswaran *et al*., 1987). In the Japanese eel, *Anguilla japonica*, however, the bioaccumulation of Cd into bone was lower than that into the kidney, liver, and gills (Yang and Chen, 1996). In rainbow trout, *Oncorhynchus mykiss*, Cd also accumulates in the liver and spleen rather than in the bones (Cammuso *et al*., 1995). In fish, the influences of Cd on bone cells such as osteoclasts (bone resorption cells) and osteoblasts (bone formation cells) have not yet been clarified.

It is known that fish scales are calcified tissue that contains osteoclasts and osteoblasts (Bereiter–Hahn and Zylberberg, 1993; Suzuki *et al*., 2016; Yoshikubo *et al*., 2005), and it has been reported that scales are a better potential internal calcium reservoir than vertebral bone, jaws, and otolithes based on studies of the ^{45}Ca–labelled calcified tissues of goldfish and killifish (Mugiya and Watabe, 1977). These facts indicate that fish scales can be utilized as a bone model of fish for analyzing heavy metals such as Cd.

On the other hand, we have developed an original

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bioassay system using goldfish scale (Suzuki *et al*., 2000; Suzuki and Hattori, 2002) because the scale is a very active organ of calcium regulation in fish. In our bioassay system, the activities of tartrate–resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) as respective indicators of each activity in osteoclasts and osteoblasts sensitively responded to calcium–regulating hormones such as calcitonin (Suzuki *et al*., 2000), parathyroid hormone (Suzuki *et al*., 2011), and prostaglandin E_2 (Omori *et al*., 2012). Furthermore, as Cd inhibited fin regeneration in tilapia fish, *Oreochromis mossambicus* (Verma, 2005), we suggested that Cd influences bone formation in fish. In the present study, therefore, we examined the effect of environmental Cd on osteoclasts and osteoblasts in an *in vivo* experiment using our original bioassay system with goldfish.

MATERIALS AND METHODS

Animals

To examine the effects of Cd on calcium metabolism, immature goldfish (*Carassius auratus*), in which the endogenous effects of sex steroids are negligible, were used in an *in vivo* study. Therefore, immature goldfish (n=32, 5.32±0.30 g) purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) were acclimated for 1 week and then used in the present study.

All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

Goldfish exposure to Cd

Goldfish were kept at 25°C during the experiments. The goldfish were randomly divided into two groups (control and experimental groups, each n=16), and each was kept in an 8 L glass aquarium. We purchased $CdCl_2$ (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and solubilized it in the environmental water at 10^{-7} M, as compared with a control group kept in Cd–free tap water. After exposure, the scales were taken from the goldfish by forceps under anesthesia with ethyl 3–aminobenzoate, methanesulfonic acid salt (Sigma–Aldrich, Inc., St. Louis, MO, USA) on days 2 and 4 (each n=8).

Measurement of TRAP activity in goldfish scales

On days 2 and 4 after $CdCl_2$ exposure, the collected scales were fixed in 10% formalin in a 0.05 M cacodylate buffer (pH 7.4) and then rinsed in distilled water. These scales were kept in 0.05 M cacodylate buffer at 4°C until analysis.

The methods for measuring TRAP activity, an indicator of osteoclasts (Suda *et al*., 1999; Vaes, 1988), have been described by Suzuki and Hattori (2002). We detected the respective enzyme activity from one scale by transferring each scale into the well of a 96–well–microplate and directly incubating it with the substrate in each well. The procedure for measuring TRAP was as follows. Each scale was transferred to its own well in a 96–well microplate after their scale weight was measured. An aliquot of 200 μl of 10 mM para–nitrophenyl–phosphate (pNPP) and 20 mM tartrate in a 0.1 M sodium acetate buffer (pH 5.3) was put into each respective well. This plate was then incubated at 20°C for 30 min while being shaken. After incubation, the reaction was stopped by adding 50 μl of 2 N NaOH. One hundred and fifty μl of a colored solution was moved to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of produced para–nitrophenol (pNP) using a standard curve for pNP. Each value was normalized by the weight of the respective scale, and their TRAP activities were then calculated. The respective mean for TRAP (obtained from three individual scales of one goldfish) was compared with that of the control group. The results are shown as means ± SEM of eight individuals.

Measurement of ALP activity in goldfish scales

On days 2 and 4 of $CdCl_2$ treatments, the scales were removed from the body of goldfish under anesthesia with ethyl 3–aminobenzoate, methanesulfonic acid salt (Sigma–Aldrich). Using these collected scales, we examined the effects of $CdCl_2$ on osteoblasts with ALP as an indicator of osteoblasts (Dimai *et al*., 1998; Suda *et al*., 1999). The scales were fixed in 10% formalin in a 0.05 M cacodylate buffer (pH 7.4) and then rinsed in distilled water. These scales were kept in a 0.05 M cacodylate buffer at 4°C until analysis. The ALP activity was measured as follows.

Each scale was transferred to its own well in a 96–well microplate. An aliquot of 200 μL of 10 mM para–nitrophenyl–phosphate in an alkaline buffer (100 mM Tris–HCl, pH 9.5; 1 mM $MgCl_2$) was added to each well. Then, this plate was incubated at 20°C for 30 min while being shaken. After incubation, the reaction was stopped by adding 50 μL of 2 N NaOH. The 150 μL of colored solution was transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted to the amount of pNP produced using a standard curve for pNP. Each value was normalized by the weight of the respective scale, and their ALP activities were then calculated (Suzuki and Hattori, 2002). The mean for ALP (obtained from three individual scales of one goldfish) was compared with that of the control group. The results are shown as means ± SEM of eight individuals.

Statistical analysis

The statistical significance between the control and $CdCl_2$–treated groups was assessed by the Student's *t*-test. The significance level chosen was $P<0.05$.

RESULTS

Effect of $CdCl_2$ on TRAP activity in goldfish scales

Goldfish were kept in tap water containing $CdCl_2$ (10^{-7} M) for 2 days. TRAP activity in the scales of goldfish decreased significantly ($P<0.05$) (Fig. 1). With 4 days of exposure to $CdCl_2$, TRAP activity also was inhibited significantly ($P<0.05$) (Fig. 2).

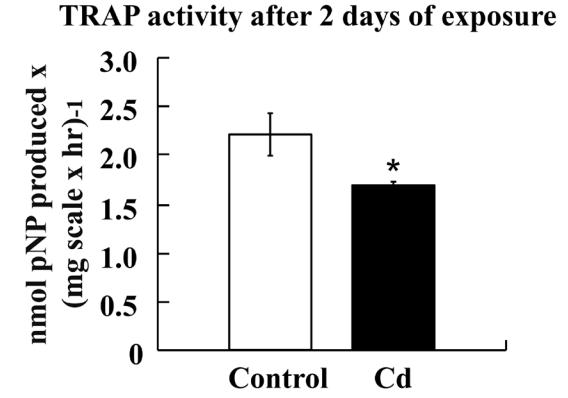

Fig. 1. Effect of $CdCl_2$ (10^{-7} M) on TRAP activity in the scales of goldfish after 2 days of exposure. * indicates a statistically significant difference at $P<0.05$ from the values in the control scales.

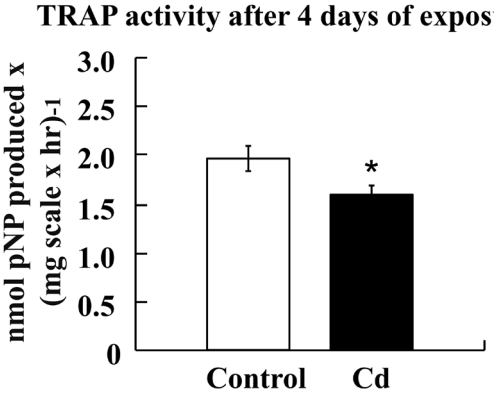

Fig. 2. Effect of $CdCl_2$ (10^{-7} M) on TRAP activity in the scales of goldfish after 4 days of exposure. * indicates a statistically significant difference at $P<0.05$ from the values in the control scales.

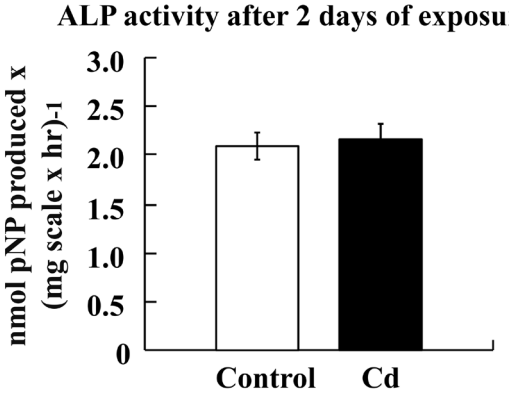

Fig. 3. Effect of $CdCl_2$ (10^{-7} M) on ALP activity in the scales of goldfish after 2 days of exposure. There was no significant differences between $CdCl_2$-treated scales and control scales.

Effect of $CdCl_2$ on ALP activity in goldfish scales

The ALP activity in $CdCl_2$–treated goldfish scales was not different from the control group after 2 days of exposure (Fig. 3). However, by 4 days after exposure, the ALP activity in the $CdCl_2$–treated goldfish scales had decreased significantly ($P<0.01$) (Fig. 4).

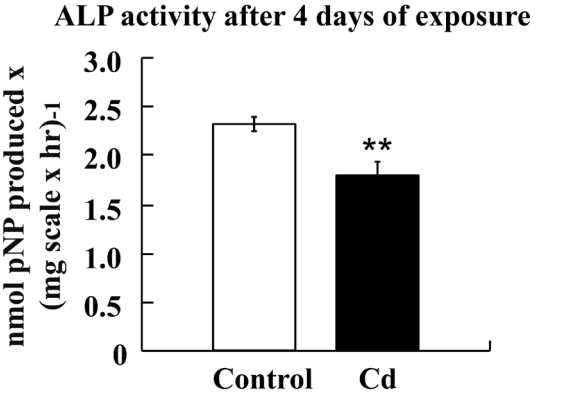

Fig. 4. Effect of $CdCl_2$ (10^{-7} M) on ALP activity in the scales of goldfish after 4 days of exposure. ** indicates a statistically significant difference at $P<0.01$ from the values in the control scales.

DISCUSSION

The present study demonstrated that osteoclasts and osteoblasts of goldfish scales sensitively responded to $CdCl_2$ in an *in vivo* experiment. Goldfish were kept in tap water containing $CdCl_2$ (10^{-7} M) for 2 days. TRAP activity in the scales of goldfish had decreased significantly ($P<0.05$) by 2 days after exposure (Fig. 1). This inhibitory effect for osteoclasts continued even after 4 days of exposure to $CdCl_2$. After 4 days of exposure to $CdCl_2$, TRAP activity also was inhibited significantly ($P<0.05$) (Fig. 2). In osteoblasts, the scale of ALP activity in $CdCl_2$–treated goldfish was no different from the control group after 2 days of exposure (Fig. 3). However, the scale ALP activity in $CdCl_2$–treated goldfish decreased significantly ($P<0.01$) by the fourth day of exposure (Fig. 4). Thus, this is the first *in vivo* experiment to indicate that both osteoclasts and osteoblasts in fish kept in tap water containing $CdCl_2$ (10^{-7} M) were suppressed. In an *in vitro* experiment, we previously reported that Cd (10^{-13} to 10^{-6} M) significantly decreased the TRAP activities of the scales at 6 hrs (Suzuki *et al.*, 2004). In the case of osteoblasts using cultured goldfish scales, the ALP activities decreased significantly with Cd treatments (10^{-7} M) for 64 and 96 hrs of incubation from the value of the control scales, although their activities did not change after 6, 18, or 36 hrs of incubation (Suzuki *et al.*, 2004). These *in vitro* toxicological data of Cd supported our present *in vivo* experiment.

On the other hand, we previously reported that the mRNA expression of metallothionein (MT), which is a metal–binding protein that protects an organism from heavy metal (Hamer, 1986; Klaassen *et al.*, 1999), increased in the Cd–treated cultured scales (Suzuki *et al.*, 2004; Suzuki *et al.*, 2006). In our *in vivo* experiment 2 days after exposure to $CdCl_2$, therefore, osteoblasts in goldfish scales were resistant to Cd, possibly resulting from the production of MT. Therefore, in the next experiment, we plan to examine the expression of MT in the scales of goldfish kept in tap water containing $CdCl_2$. The resistance of Cd in osteoblasts will be examined using this bioassay system.

Teleost fish have quite unique hard tissue and scales that possess osteoclasts (bone resorption cells), osteoblasts (bone formation cells), and calcified bone matrix, including type 1 collagen, bone γ–carboxyglutamic acid protein, and osteonectin (Bereiter–Hahn and Zylberberg, 1993; Suzuki and Hattori, 2002; Suzuki *et al*., 2008; Thamamongood *et al*., 2012; Redruello *et al*., 2005). In all vertebrates, including teleosts, blood calcium levels are strictly maintained at a constant level (around 2 to 3 mM) (Suzuki *et al*., 2016). Teleost scales have important functions in controlling blood calcium levels because teleost scales, having both osteoblasts and osteoclasts, are known to function as potential internal calcium storage and are very similar to those in the mammalian bones (Persson *et al*., 1998; Suzuki *et al*., 2008). Particularly, in fresh water teleosts, such as goldfish, which live in a low–calcium state, the scales have remarkably important roles in regulating blood calcium concentrations. The above facts indicate that teleost scale is a suitable model for analyzing bone metabolism in fish. The bioassay system using these characteristic scales seems to be useful for analyzing environmental pollutants. Using this *in vitro* system, actually, the influences of endocrine disrupters, such as bisphenol–A (Suzuki and Hattori, 2003), tributyltin (TBT) (Suzuki *et al*., 2006), and polychlorinated biphenyl (Yachiguchi *et al*., 2014) on osteoblasts and osteoclasts have been examined in addition to Cd. Furthermore, we recently demonstrated the toxicity of gadolinium (Gd) to osteoclasts and osteoblasts of goldfish scales (Suzuki *et al*., 2019). Even Gd of 10^{-13} M suppressed osteoclastic activity after 6 hours of incubation. Also, the osteoblastic inhibition of Gd (10^{-10} to 10^{-6} M) was almost equal to that of TBT (10^{-10} to 10^{-5} M) (Suzuki *et al*., 2019). Considering the above facts together with the present data regarding Cd, our scale bioassay system that can be used both *in vivo* and *in vitro* will be a useful tool for analyzing the effects of environmental pollutants on bone metabolism.

AUTHOR CONTRIBUTIONS

N. Suzuki, T. Sekiguchi, N. Shimizu, Y. Sasayama, Y. Tabuchi, Y. Shimasaki, and Y. Oshima designed the study. N. Suzuki, U. Kawago, M. Honda, A. K. Srivastav, T. Amornsakun, K. Matsumoto, H. Matsubara, J. Hirayama, A. Hattori, Y. Shimasaki, and Y. Oshima performed experiments, analyzed the data, and wrote the paper. Y. Oshima and Y. Shimasaki supervised the work. All authors assisted in editing the manuscript and approved the final version.

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